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Report

NudC Is Required for Plk1 Targeting to the Kinetochore and Chromosome Congression

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Summary

The equal distribution of chromosomes during mitosis is critical for maintaining the integrity of the genome. Essential to this process are the capture of spindle microtubules by kinetochores and the congression of chromosomes to the metaphase plate [1]. Polo-like kinase 1 (Plk1) is a mitotic kinase [2] that has been implicated in microtubule-kinetochore attachment, tension generation at kinetochores, tension-responsive signal transduction, and chromosome congression [3–7]. The tension-sensitive substrates of Plk1 at the kinetochore are unknown. Here, we demonstrate that human *Nu*clear *d*istribution protein *C* (NudC), a 42 kDa protein initially identified in *Aspergillus nidulans* [8, 9] and shown to be phosphorylated by Plk1 [10], plays a significant role in regulating kinetochore function. Plk1-phosphorylated NudC colocalizes with Plk1 at the outer plate of the kinetochore. Depletion of NudC reduced end-on microtubule attachments at kinetochores and resulted in defects in chromosome congression at the metaphase plate. Importantly, NudC-deficient cells exhibited mislocalization of Plk1 and the Kinesin-7 motor CENP-E from prometaphase kinetochores. Ectopic expression of wild-type NudC, but not NudC containing mutations in the Plk1 phosphorylation sites, recovered Plk1 localization at the kinetochore and rescued chromosome congression. Thus, NudC functions as both a substrate and a spatial regulator of Plk1 at the kinetochore to promote chromosome congression.

Results and Discussion

Plk1-Phosphorylated NudC at the Outer Plate of Kinetochores

NudC binds directly to Plk1 and is phosphorylated by Plk1 during mitosis on two highly conserved serine residues (Ser-274 and Ser-326 in human NudC) in the carboxyl terminus [10]. We determined the intracellular localization of phospho-NudC by using anti-phosphoserine antibodies against Ser-326 of NudC (Figure 1A). The phospho-Ser-326-NudC (pSer-326-NudC) antibody strongly recognized NudC in lysates from mitotic HeLa cells in a phosphorylation-dependent manner, but not in lysates from asynchronous cells (see Figure S1 in the Supplemental Data available with this article online). Using indirect immunofluorescence microscopy, we detected phospho-NudC primarily at the kinetochores of HeLa cells in prometaphase, metaphase, and anaphase (Figures 1B and 1C). Phospho-NudC at the kinetochores was largely reduced in telophase (data not shown). Notably, phospho-NudC colocalized with Plk1 at the kinetochores (Figures 1C and 1D). We further compared the localization of phospho-NudC relative to other centromere and kinetochore proteins and found that phospho-NudC flanked the centromeric regions occupied by the CREST (*c*alcinosis, *R*aynaud's phenomenon, *e*sophageal dismotility, *s*clerodactyly, *t*elangiectasia) antigens and the chromosomal passenger protein Aurora B kinase (Figure 1D). Interestingly, phospho-NudC was flanked by p150 dynactin (Figure 1D), which localizes to the corona region external to the outer plate of the kinetochore [11]. This staining pattern suggests that phospho-NudC localizes to the outer plate of the kinetochore. Furthermore, in cells depleted of Plk1 by small interfering RNAs (siRNAs), NudC phosphorylation at Ser-326 was abrogated as shown by immunoblotting (Figure 1E) and staining at the kinetochore (Figure 1F), confirming that Ser-326 in NudC is phosphorylated by Plk1 in vivo. Together, these results show that Plk1 phosphorylates NudC at the outer plate of kinetochores in the early stages of mitosis.

Phospho-NudC Accumulates on Kinetochores with Reduced Tension

Recent studies have shown that Plk1 may mediate a tension-responsive signaling pathway at kinetochores [3, 4]. Consistent with this idea, we observed a higher level of Plk1 staining at kinetochores in prometaphase than in metaphase cells (Figure S2A) because of the lower level of interkinetochore tension during prometaphase. Phospho-NudC staining was similarly greater on prometaphase than on metaphase kinetochores, suggesting that the localization of Plk1-phosphorylated NudC to kinetochores is regulated by tension (Figure S2A). To determine whether the presence of phospho-NudC at the kinetochore is sensitive to tension, we used the microtubule-stabilizing drug taxol to disrupt kinetochore tension without affecting microtubule-kinetochore attachment (Figure S2B). In this manner, taxol has been shown to induce the enrichment of proteins that are responsive to a reduction in tension at the kinetochore [3, 12, 13]. HeLa cells were first treated with monastrol, an Eg5 kinesin motor (Kinesin-5) inhibitor [14] that reversibly arrests cells in mitosis with monopolar spindles [15]. Cells were released from monastrol, enriched in metaphase by incubation in media containing the proteasome

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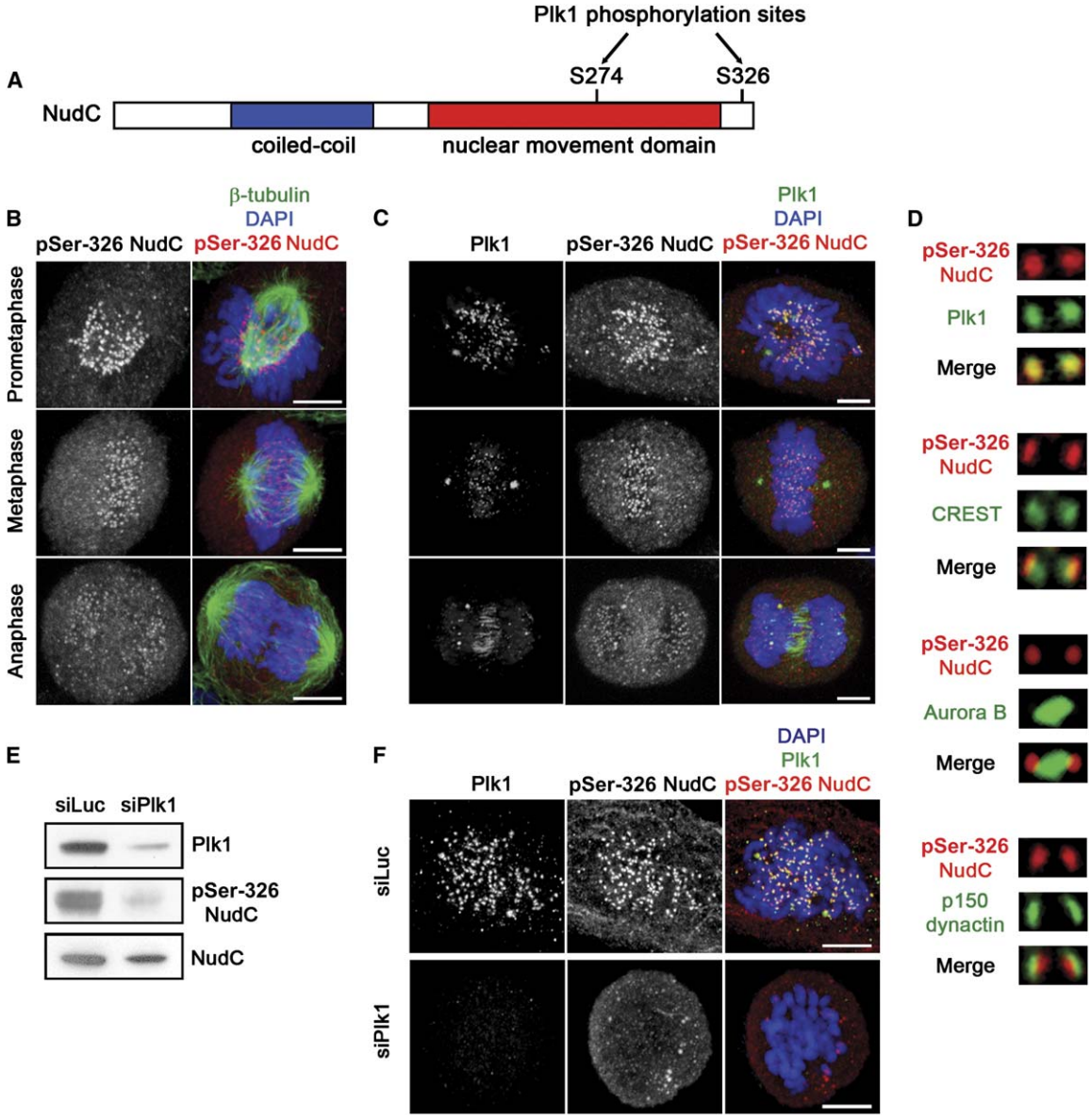

Figure 1. Phospho-NudC Colocalizes with Plk1 at the Outer Kinetochore Plate in Prometaphase

(A) NudC has an N-terminal coiled-coil domain (blue), a highly conserved C-terminal 154 amino acid nuclear movement domain [33] (red), and two serine residues (S274 and S326 in human NudC) that have been shown to be phosphorylated by Plk1 [10]. A phosphospecific antibody against the phosphorylated Ser-326 residue on NudC was developed (Figure S1).

(B) HeLa cells were stained for pSer-326-NudC (red), tubulin (green), and DNA (blue). Phospho-NudC staining at the kinetochores in prometaphase, metaphase, and early anaphase cells is shown in black and white for contrast. Scale bars represent 5 μm.

(C) HeLa cells were stained for pSer-326-NudC (red), Plk1 (green), and DNA (blue). Scale bars represent 5 μm.

(D) Enlargements of kinetochore pairs stained for pSer-326-NudC (red) and one of the following are shown: Plk1 (green), CREST (green), Aurora B (green), or p150 dynactin (green). The kinetochore pair stained for pSer-326-NudC and p150 was taken from a HeLa cell treated for 14 hr with 100 ng/ml nocodazole. Optical sections, enlarged 7-fold from images acquired with the same magnification as those shown in (C), are shown.

(E) Depletion of Plk1 abrogates Ser-326 NudC phosphorylation in vivo. Lysates from luciferase or Plk1 siRNA-treated HeLa cells were prepared 48 hr after siRNA transfection and immunoblotted with Plk1, pSer-326-NudC, or NudC (2D9) antibodies.

(F) Depletion of Plk1 abrogates the pSer-326-NudC epitope at kinetochores in vivo. HeLa cells transfected with luciferase or Plk1 siRNAs for 48 hr were stained for Plk1 (green), phospho-NudC (red), and DNA (blue). Scale bars represent 5 μm.

inhibitor MG132, and subsequently treated in media with or without taxol. Taxol treatment of metaphase cells induced a 2-fold increase in phospho-NudC staining at kinetochores (Figure S2B), indicating that phospho-NudC accumulation at the kinetochore was induced by a loss of tension. At present, it is unclear whether the concomitant reduction of phospho-NudC and Plk1 from kinetochores in metaphase cells (Figures 1B and 1C; also Figure S2) and in Plk1 siRNA cells (Figure 1F) represents dissociation of NudC from kinetochores, dephosphorylation of phospho-NudC, or both. The localization of phospho-NudC to the outer kinetochore (Figure 1D) in a tension-sensitive manner (Figure S2) suggests a novel role for NudC in regulating kinetochore function.

Phospho-NudC Is Required for Chromosome Congression

To test if NudC is required for kinetochore function, we depleted endogenous NudC by siRNA in mitotically synchronized HeLa cells. Transfection of two distinct siRNAs targeting NudC efficiently reduced NudC levels, whereas control siRNAs targeting firefly luciferase did not (Figure 2A). As an initial indicator of kinetochore function, chromosome congression was examined in cells released from a monastrol blockade. HeLa cells were transfected with siRNAs, treated with monastrol for 4 hr, and released for 1 hr into growth medium supplemented with MG132 (Figure 2B). In control cells (n = 223), 73% (±2% SD) of mitotic cells exhibited complete chromosome alignment at the metaphase plate (Figure 2B). In contrast, only 19% (±6% SD) of NudC-deficient cells at mitosis (n = 142) demonstrated chromosomes that were fully congressed at the metaphase plate (Figure 2B). In a majority of NudC-deficient cells (81% ±6% SD, n = 142), one or more chromosomes failed to align at the metaphase plate (Figure 2B). Thus, NudC depletion resulted in a significant increase in chromosome misalignment at the metaphase plate.

To test if NudC phosphorylation by Plk1 is required for proper chromosome alignment at the metaphase plate, we performed ectopic rescue experiments of NudC depletion with EGFP-tagged, siRNA-resistant (r) forms of either wild-type NudC (NudC-WTr) or phospho-defective mutant NudC in which Ser-274 and Ser-326 were mutated into alanine residues (NudC-AAr). Immunoblot analysis showed that EGFP-NudC-WTr and EGFP-NudC-AAr were resistant to siRNA-mediated NudC knockdown, whereas the levels of endogenous NudC were substantially reduced in cells (Figure 2C). For the rescue experiment, cells were treated with siRNAs, transfected with either EGFP-NudC-WTr or EGFP-NudC-AAr, and arrested in metaphase after release from a monastrol-induced mitotic blockade (Figure 2D). Chromosome-congression defects were scored in EGFP-positive cells. In luciferase-siRNA cells transfected with an EGFP vector alone, 61% (±6% SD) of the cells showed fully aligned chromosomes at the metaphase plate (Figure 2D). Consistent with the results in Figure 2B, NudC depletion reduced the percentage of cells with fully aligned chromosomes to 31% (±3% SD) (Figure 2D). Ectopic expression of EGFP-NudC-WTr restored proper chromosome congression to the level observed in control cells (Figure 2D). However, expression of the phospho-defective EGFP-NudC-AAr mutant did not rescue the chromosome-misalignment phenotype (Figure 2D). Similar results were obtained when the rescue experiment was conducted in cells released from nocodazole instead of monastrol blockade (Figure S3). These studies suggest that chromosome alignment at the metaphase plate requires not only a proper level of NudC but also phosphorylation of NudC by Plk1.

NudC Promotes End-On Kinetochore-Microtubule Attachments

We tested the possibility that the defect in chromosome alignment in NudC-deficient cells might be due to problems with microtubule attachment to the kinetochore. To determine whether NudC-deficient cells are able to establish stable kinetochore-microtubule attachments, we analyzed HeLa cells exposed to cold temperatures. Cold treatment selectively destabilizes nonkinetochore microtubule fibers and permits clear examination of microtubule occupancy at kinetochores [16]. siRNA-transfected HeLa cells were arrested in metaphase and briefly placed in an ice bath prior to fixation. In control cells at metaphase, spindle microtubules remained intact and demonstrated bipolar attachment to kinetochore pairs (Figure 3A). In contrast, NudC-deficient cells exhibited a range of spindle morphologies after cold treatment. Many of the cells demonstrated reduced cold-stable kinetochore-microtubule fibers (Figure 3B; also Figure S4A), suggesting a reduction in stable attachments between kinetochores and spindle microtubules. Examination of confocal slices through these cells revealed that 82% (±7% SD) of kinetochores in control cells had end-on attachments to spindle microtubules (Figures 3A [single optical section shown in inset] and 3C). In contrast, in NudC-deficient cells, end-on kinetochore-microtubule attachments were reduced to 63% (±9% SD), with a concomitant increase in kinetochores that were attached in a side-on manner to microtubules (28% ± 6% SD, compared to 12% ± 6% SD for controls) (Figures 3B [single optical sections shown in insets 1 and 2] and 3C; also Figure S4A). There was little change between control and NudC-deficient cells in the number of kinetochores without microtubule attachments (Figure 3C).

Cells require stable, bipolar attachment between microtubules and kinetochores to produce pulling forces across sister kinetochores [17]. As an additional test for the stability of microtubule-kinetochore attachments, we examined the ability of siRNA-transfected cells to generate tension across sister kinetochores by measuring the interkinetochore distance between paired kinetochores in the same confocal plane [18]. By this measurement, NudC-deficient cells showed a significant decrease in interkinetochore tension generation compared to that of the controls, with the reduced tension comparable to that observed in taxol- or nocodazole-treated cells (Figure S4B). Together, these results suggest that a proper level of NudC is required for stable end-on microtubule-kinetochore attachments and the generation of tension across sister kinetochores.

NudC Promotes the Targeting of Plk1 and CENP-E to Kinetochores

Localized Plk1 activity is important for microtubule-kinetochore attachment and chromosome congression [19]. To determine whether NudC is involved in targeting Plk1 to the kinetochore, we analyzed Plk1 localization in NudC-deficient cells. In control cells, Plk1 and phospho-NudC clearly colocalized at kinetochores (Figure 4A). In NudC-deficient cells, Plk1 was no longer enriched at kinetochores (Figure 4A, middle panel). Plk1 staining was diffuse and occasionally overlaid the entire chromosome (Figure 4A, lower panel). The lack of discrete Plk1 localization at kinetochores in NudC-deficient cells suggests that NudC is necessary for the proper targeting of Plk1 to kinetochores. To further investigate whether a proper level of NudC as well as Plk1 phosphorylation of NudC was important for targeting Plk1 to kinetochores, we transfected NudC-deficient cells with either EGFP-NudC-WTr or EGFP-NudC-AAr. Both EGFP and

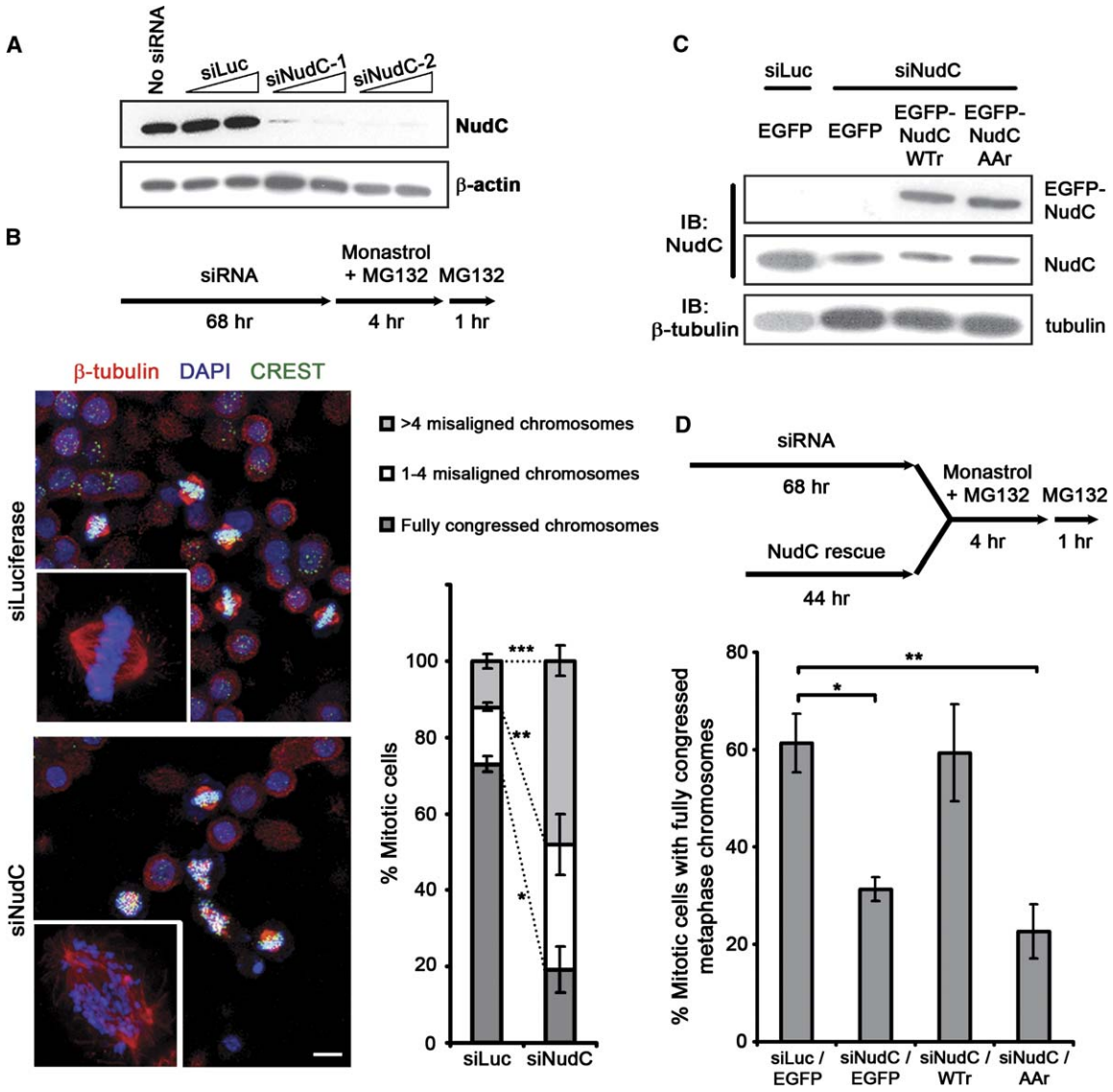

Figure 2. NudC Levels and Plk1-Phosphorylated NudC Are Important for Chromosome Congression

(A) Immunoblot analysis of NudC depletion. HeLa cells were transfected for 72 hr with increasing concentrations (100 nM, 200 nM) of luciferase siRNA (siLuc) or either of two siRNA oligonucleotides targeting different regions of NudC (siNudC-1 and siNudC-2). Immunoblots with NudC (70/1) and actin antibodies are shown.

(B) Effect of NudC levels on chromosome congression during metaphase. Cells transfected with control or NudC siRNAs were enriched in mitosis with monastrol, released into MG132 to arrest cells in metaphase, and stained for kinetochores (CREST, green), tubulin (red), and DNA (blue). Insets show a 3-fold enlargement of representative control and NudC siRNA cells (stained for tubulin and DNA). The percentage of MG132-arrested control (n = 223) or NudC siRNA (n = 142) cells with fully congressed chromosomes, one to four misaligned chromosomes, or greater than four misaligned chromosomes were scored. Graph shows the average (±SD) of three independent experiments. *p = 0.0001. **p = 0.015. ***p = 0.0002. The scale bar represents 10 μm.

(C) Immunoblot analysis of NudC in NudC-deficient cells that have been transfected with an empty EGFP vector or with EGFP-tagged, siRNA-resistant (denoted by "r") NudC constructs bearing five silent mutations in the siRNA-targeted region. NudC and EGFP-NudC are approximately 42 kDa and 69 kDa, respectively. NudC (2D9) and NudC (70/1) antibodies were used to detect ectopic and endogenous NudC, respectively.

(D) Rescue of chromosome misalignment in NudC-deficient cells by wild-type but not Plk1-phospho-defective NudC. Cells were depleted of NudC by siRNA, transfected with siRNA-resistant NudC constructs (shown in [C]) 24 hr after siRNA treatment, and processed as indicated. The percentage of EGFP-positive MG132-arrested cells with fully congressed chromosomes was determined. Data are the average (±SD) of three independent experiments, in which more than 100 EGFP-positive cells were counted per experiment. *p = 0.0014. **p = 0.0012.

EGFP-NudC showed diffuse cytosolic localization (Figure 4B). As shown in Figure 4A, Plk1 localized properly to kinetochores in EGFP-expressing control cells but was more diffusely localized throughout the cytoplasm in NudC-deficient cells (Figure 4B). Strikingly, in NudC-deficient cells that ectopically expressed NudC-WTr, Plk1 localization to kinetochores was restored (Figure 4B). In contrast, in NudC-deficient cells reconstituted with phospho-defective NudC-AAr, Plk1 staining remained diffuse and largely cytoplasmic (Figure 4B).

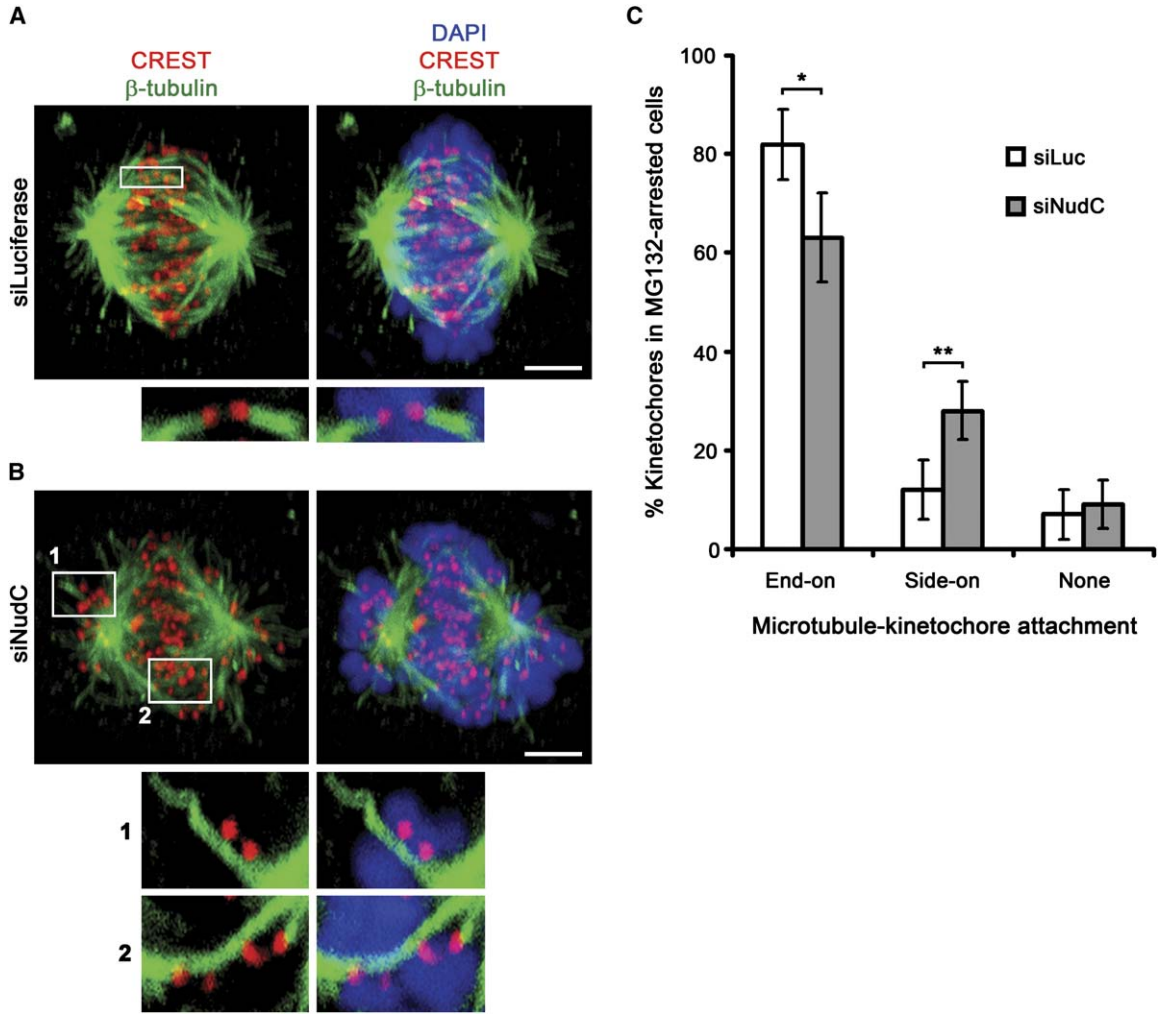

Figure 3. NudC Depletion Reduces End-On Attachments between Microtubules and Kinetochores

(A) HeLa cells were transfected with luciferase siRNAs, arrested in metaphase with MG132, and placed in an ice bath for 10 min prior to fixation. Cells were stained for kinetochores (CREST, red), tubulin (green), and DNA (blue). A single optical section of representative end-on microtubule-kinetochore attachments is shown without or with DNA overlay. Insets were enlarged 3-fold. The scale bar represents 5 μm.

(B) HeLa cells were transfected with NudC siRNAs and processed as described in (A). Insets show single optical sections depicting side-on attachments of kinetochores to spindle microtubules. The scale bar represents 5 μm.

(C) The average fraction of kinetochores (±SD) with end-on, lateral side-on, or no microtubule attachment was determined for luciferase siRNA cells (n = 18) and NudC siRNA cells (n = 20). Confocal sections were taken through the thickness of the cell, and at least 30 kinetochores were evaluated in each cell for their attachment to microtubules. *$p < 0.0001$. **$p < 0.0001$.

We further evaluated the ability of NudC-WT and NudC-AA to bind Plk1 in a GST pull-down assay. Both forms of NudC constitutively associated with Plk1 (Figure 4C). Thus, the reduction in Plk1 localization to the kinetochore in cells expressing NudC-AA is not due to Plk1's failure to bind phospho-defective NudC, but rather to the inability of Plk1 to be properly targeted to the kinetochore by phospho-defective NudC. In other words, proper targeting of Plk1 to kinetochores requires NudC phosphorylation by Plk1.

Many proteins, including the kinetochore-associated microtubule motor CENP-E, depend on Plk1 for their accumulation at the kinetochore [3]. CENP-E has been shown to be important for stable kinetochore-microtubule interactions as well as for chromosome congression [20–23]. To determine whether Plk1 mislocalization in NudC-deficient cells affects the proper targeting of CENP-E to the kinetochore, we investigated the subcellular localization of CENP-E. In control cells, CENP-E is localized to kinetochores during prometaphase (Figure 4D). In contrast, NudC-deficient cells exhibited reduced CENP-E staining on prometaphase kinetochores (Figure 4D). These results suggest that NudC is important for the proper localization of CENP-E to kinetochores and reveal one mechanism by which NudC deficiency induces defects in kinetochore-microtubule attachment and chromosome congression.

Model for NudC Function at the Kinetochore

Based on our findings, we propose a model for NudC function at the kinetochore (Figure 4E). During prometaphase, Plk1 interacts with and phosphorylates NudC, and the complex formed by Plk1 and phospho-NudC undergoes a conformation change that allows these

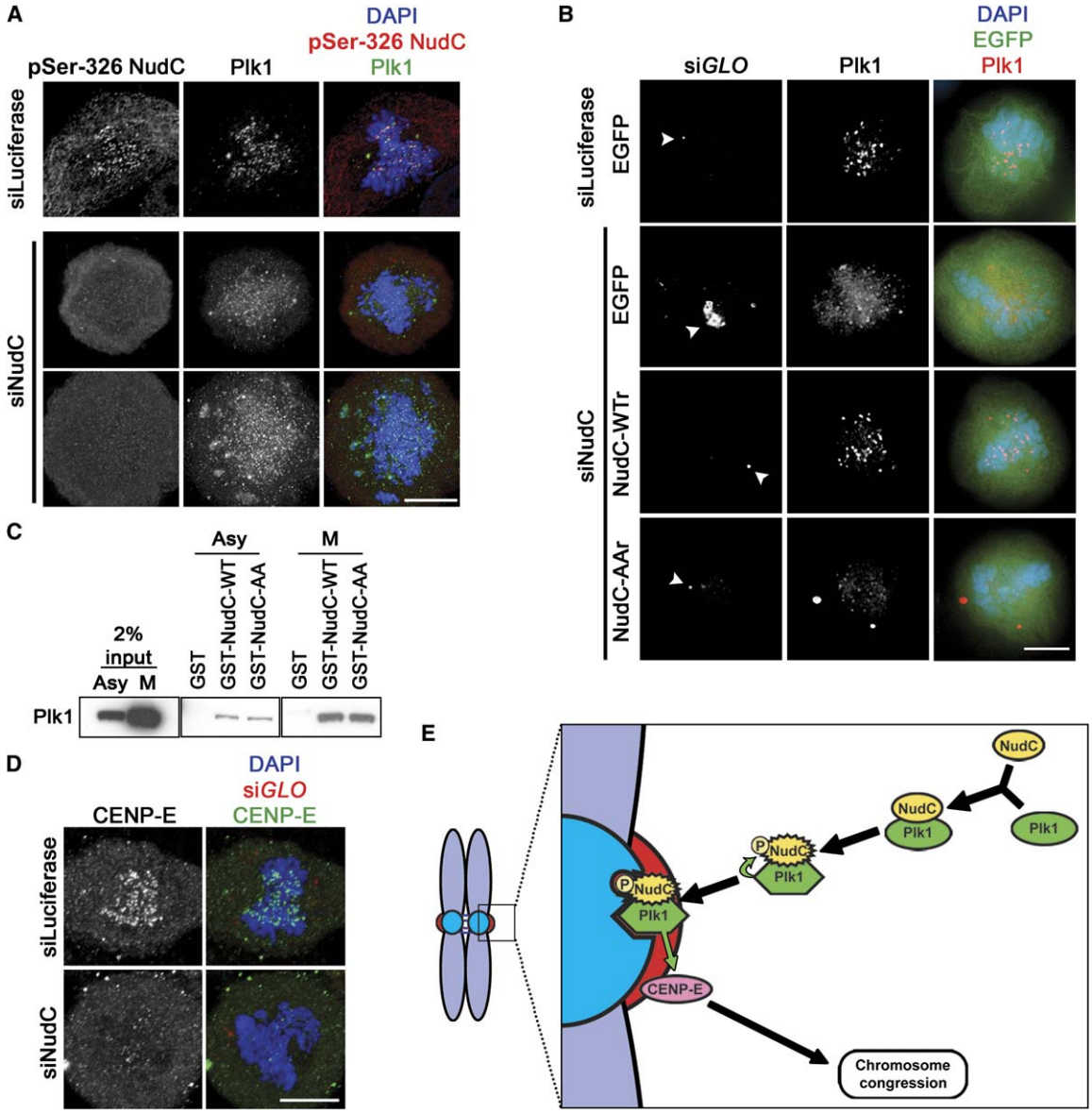

Figure 4. NudC Regulates Plk1 Localization to the Kinetochore

(A) Plk1 is mislocalized from the kinetochores of NudC-deficient HeLa cells. HeLa cells were transfected for 72 hr with control or NudC siRNAs and stained for phospho-NudC (red), Plk1 (green), and DNA (blue). Two representative NudC-deficient cells with varying levels of Plk1 mislocalization from the kinetochore are shown. The scale bar represents 10 μm.

(B) Rescue of Plk1 kinetochore localization in NudC-deficient cells by wild-type but not Plk1-phospho-defective NudC. HeLa cells were treated with luciferase or NudC siRNAs and cotransfected with a fluorescently tagged nontargeting control siRNA oligonucleotide (si*GLO*). Cells were subsequently transfected with EGFP-tagged, siRNA-resistant NudC-WTr or NudC-AAr constructs and fixed 72 hr after the initial siRNA transfection. Only si*GLO*- (shown in black and white for contrast, arrowhead) and EGFP- (green) double-positive cells were analyzed for Plk1 staining (red). DNA staining is shown in blue. The scale bar represents 10 μm.

(C) Plk1 interacts with NudC-WT and NudC-AA in a GST pull-down assay. Asynchronous (Asy) or mitotic (M) HeLa cell lysates were incubated with GST, GST-NudC-WT, or GST-NudC-AA beads. Bound proteins were probed with Plk1 antibodies.

(D) CENP-E is mislocalized from the kinetochores of NudC-deficient cells. Control or NudC siRNAs were cotransfected with si*GLO* oligonucleotides (red) into HeLa cells. Cells were fixed 72 hr after transfection and stained for CENP-E (green) and DNA (blue). si*GLO*-positive cells are shown. The scale bar represents 10 μm.

(E) Model for NudC function at the kinetochore. Both Plk1 (green) and phospho-NudC (yellow) accumulate on kinetochores with reduced tension during prometaphase. NudC phosphorylation by Plk1 is required for these two proteins to cotarget to the outer kinetochore (red). Plk1 activity at the kinetochore stimulates the kinetochore recruitment of CENP-E (pink), which promotes stable end-on microtubule-kinetochore interactions and proper congression of chromosomes (light blue) to the metaphase plate. Whether the complex formed by Plk1 and phospho-NudC interacts with other kinetochore or centromeric (blue) protein(s) is currently unknown.

proteins to cotarget to the outer kinetochore. Localized Plk1 activity at the kinetochore is important for the proper kinetochore accumulation of CENP-E, which promotes stable kinetochore-microtubule interactions and proper chromosome congression to the metaphase plate. We suggest that in the absence of NudC, Plk1 is unable to localize to the kinetochore and is therefore unable to regulate kinetochore functions. Recent studies have shown that two other proteins, the checkpoint kinase Mps1 [4] and the chromosomal passenger protein INCENP [24], also play a role in the recruitment of Plk1 to kinetochores. Whether NudC functions in the same or distinct pathways as these proteins for targeting Plk1 to kinetochores, and whether phospho-NudC plays additional roles at the kinetochore remain to be determined.

Recent studies have suggested that Plk1 regulates kinetochore assembly and spindle checkpoint signaling at untensed kinetochores by creating a tension-sensitive phosphoepitope recognized by the 3F3/2 monoclonal antibody [3, 4]. Because both NudC and the 3F3/2 antigen appear to be phosphorylated by Plk1 in a tension-sensitive manner (this study;) [3, 4], we examined whether NudC might be one of the Plk1-phosphorylated antigens recognized by the 3F3/2 antibody. Preliminary experiments show that NudC does not co-immunoprecipitate with the 3F3/2 antibody (M.N., Y.K., and L.Y. Y-L., unpublished observations; G.J. Gorbsky, personal communication). Therefore, 3F3/2 phosphoepitope generation and NudC phosphorylation by Plk1 at the kinetochore are likely to be separate events.

Our previous studies have shown that NudC is a dynein motor-associated factor that colocalizes with dynein, dynactin, and Lis1 at the centrosome and cell cortex [10, 25, 26]. However, Plk1-phosphorylated NudC does not fully colocalize with p150 dynactin in the fibrous corona of kinetochores (Figure 1D), and NudC deficiency only partially phenocopies aspects of dynein and Lis1 disruption [27–29]. These observations suggest that phospho-NudC performs functions at the kinetochore that are independent of dynein and dynactin. The precise relationships among NudC, Plk1, and the dynein-dynactin motor complex over cell division remain to be determined.

NudC Is a Substrate and Spatial Regulator of Plk1

Our studies reveal a novel role for phospho-NudC as a spatial regulator of Plk1 at the kinetochore. In support of this idea, we and others have previously suggested a role for Plk1 substrates such as NudC in targeting Plk1 to the centrosome and midbody [30, 31]. It is currently unclear whether the chromosome-congression errors observed in early mitosis (this study) contribute to cleavage furrow regression, cytokinesis failure, and multinucleation phenotype previously observed in NudC-deficient cells [10, 30]. Studies by others indicate that chromosome nondisjunction can lead to cleavage furrow regression and the formation of binucleate cells [32]. However, the localization of NudC to the midzone and midbody [10, 30], as well as the NudC-dependent targeting of Plk1 to these structures [30], raises the possibility that the Plk1-NudC complex functions during cytokinesis independently of its role at the kinetochore. Thus, NudC is a substrate and a spatial regulator of Plk1 at multiple mitotic structures. Taken as a whole, our findings underscore the importance of NudC in the preservation of genomic integrity at multiple points during mitosis.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and four figures that can be found with this article online at http://www.current-biology.com/cgi/content/full/16/14/1414/DC1/.

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Note Added in Proof

The paper "Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1" by W. Qi, Z. Tang, and H. Yu describes Bub1 as another protein critical for the localization of Plk1 to kinetochores (Mol. Biol. Cell., published online June 7, 2006).